Development of 32 novel microsatellite loci in Juglans sigillata (Juglandaceae) using genomic data

Applications in Plant Sciences

PRIMER NOTE

Development of 32 novel microsatellite loci in *Juglans sigillata* using genomic data

Zu-Chang Xu[1,2,3], Ye-Chuan Jin[4], Richard I. Milne[5], Zuo-Ying Xiahou[2], Han-Tao Qin[1], Lin-Jiang Ye[1], Lian-Ming Gao[1], Jie Liu[1,2,6], and De-Zhu Li[2,6]

Manuscript received 31 October 2019; revision accepted 19 December 2019.

[1] CAS Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, Yunnan, China

[2] Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, Yunnan, China

[3] University of the Chinese Academy of Sciences, Beijing 100049, China

[4] School of Life Sciences, Yunnan University, Kunming 650091, Yunnan, China

[5] Institute of Molecular Plant Sciences, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JH, United Kingdom

[6] Authors for correspondence: liujie@mail.kib.ac.cn; dzl@mail.kib.ac.cn

Citation: Xu, Z.-C., Y.-C. Jin, R. I. Milne, Z.-Y. Xiahou, H.-T. Qin, L.-J. Ye, L.-M. Gao, J. Liu, and D.-Z. Li. 2020. Development of 32 novel microsatellite loci in *Juglans sigillata* using genomic data. *Applications in Plant Sciences* 8(3): e11328.

doi:10.1002/aps3.11328

PREMISE: A novel set of microsatellite markers was developed for *Juglans sigillata* (Juglandaceae), an endemic walnut species in southwestern China, to facilitate cultivar identification and future investigations into the genetic structure and domestication history of this species and its close relatives.

METHODS AND RESULTS: We developed 32 microsatellite loci for *J. sigillata* using genomic data and used them to examine 60 individuals from three natural populations. A high level of polymorphism was detected by these primers, with up to eight alleles observed per locus, and an average of four alleles across populations. The levels of observed and expected heterozygosity ranged from 0.000–1.000 and 0.000–0.785, respectively. All but two of the loci were also successfully amplified in three closely related Eurasian *Juglans* species (*J. regia*, *J. cathayensis*, and *J. mandshurica*).

CONCLUSIONS: The microsatellite loci identified here provide a powerful resource for examining the genetic structure and domestication history of *Juglans*, as well as identification of its cultivars.

KEY WORDS genomic data; Juglandaceae; *Juglans sigillata*; microsatellite; southwestern China; walnut.

Juglans sigillata Dode (Juglandaceae), the iron walnut, is a woody perennial nut tree endemic to southwestern China (Lu et al., 1999). As one of the most important edible walnut species, *J. sigillata* is significantly different from the common walnut (*J. regia* L.) in morphology, fruit quality, and environmental adaptability (Lu et al., 1999; Gunn et al., 2010). There are more than 100 local cultivars and superior individuals of this species, which vary in seed quality and ecological adaptation. Therefore, *J. sigillata* is an important component of the genetic resources within *Juglans* L., given its great value for both cultivar development and scientific research. However, accurate determination of *Juglans* species requires both taxonomic expertise and complete specimens, and even then, identification can be difficult between closely related species or infraspecific taxa (e.g., cultivars), due for example to effects of age and environment. The scenario presents a major problem for improvement and conservation because accurate identification and delimitation of taxa are at the core of crop genetic breeding and genetic resource conservation (Cooke, 1995). Moreover, the precise taxonomic determination of specimens at the marketplace is rendered difficult or impossible; for example, the methods available to local people and authorities might not distinguish among dried nuts or seedlings of different *Juglans* taxa usually sold in markets.

As a traditional molecular marker, simple sequence repeats (SSRs or microsatellites) have been widely used in genetic diversity research. SSRs are abundant, genome- and locus-specific, codominantly inherited, highly reproducible, and usually highly polymorphic (Powell et al., 1996). They have extensive applications, including but not limited to forensics, population and conservation genetics, identification of species and cultivars, and phylogeography (Hodel et al., 2016). However, SSR development typically requires a substantial input of time, funds, equipment, and expertise, and the biggest challenge involved is the identification or development of high-quality primers that will amplify across the taxonomic group of interest (Zane et al., 2002). Nonetheless, once suitable primers are available, taxon identification via DNA fingerprinting becomes relatively straightforward and circumvents the need for taxonomic expertise.

A large number of *Juglans* microsatellite primers have been developed using traditional approaches (e.g., Woeste et al., 2002; Hoban et al., 2008; Ikhsan et al., 2016), and some of them have been

TABLE 1. Characteristics of 32 microsatellite loci isolated from *Juglans sigillata*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	Fluorescent dye	GenBank accession no.
JS01	F: AGGGATTGTGTTCAAATTGACCT R: GCTGGAGTGACAAATTCGAGT	$(CACGA)_5$	79–94	56	FAM	MN604168
JS02	F: GCATGATTAGTGACTTGAATTAGAAGC R: CATGACATGCTCGACACGC	$(AAAG)_8$	87–115	57	HEX	MN604165
JS03	F: TGACGAGGTTTACCAGATGGG R: CGTTCTTCTTTCAGAGTGCTGTT	$(GAA)_5$	90–105	58	TAMRA	MN604150
JS04	F: GAGGAAGAAGAGATGAAGACGA R: CCCTTTCTCTATAATCCAATGAAATCC	$(GAA)_6$	93–111	56	HEX	MN604155
JS05	F: CGGCATTACAGTCGGCAGTA R: ACAATTCCCGTGCTGCATCT	$(GAA)_{10}$	93–120	57	FAM	MN604159
JS06	F: CCCTGCATGCAATCAATCACA R: ATGGGACGAGTGATGGACTC	$(AGT)_5$	96–111	55	TAMRA	MN604154
JS07	F: ACCAGCAGTTCCATGTACGG R: GCTCATGCCATTATCTGCTTCG	$(GAG)_9$	111–132	57	TAMRA	MN604158
JS08	F: GCATGCATTGGAACCTACCC R: TCAAAGCATTAACGTTAGCGAC	$(AAGA)_6$	112–122	56	TAMRA	MN604166
JS09	F: TTCGACCGCGTTTCCAGTTA R: CCAGACTCACGGTCAGTTCC	$(TTC)_7$	116–131	56	FAM	MN604156
JS10	F: GCTCCAGGCACATGAAAGAG R: AATCCACCATTGCCCAACGA	$(GGA)_7$	121–142	57	HEX	MN604163
JS11	F: CCAGACGGGTCCAGGTAT R: GCCTTAGAGGTCAAGAATCCGA	$(AGC)_8$	123–141	57	FAM	MN604149
JS12	F: TCAACATTGGCGAGGTGACA R: AGGCAAGTCTACTTCTTTCCCT	$(TTA)_7$	128–155	55	TAMRA	MN604152
JS13	F: TCTTGTCAGCATACTAAGCTTGTT R: ACTAACTGCATATAGGATCAACCA	$(TTCT)_5$	129–158	56	HEX	MN604164
JS14	F: CACATCGAGTGTTTCAAGTGACA R: TGCACATGAGGAATTAACTGCTT	$(TGC)_6$	134–149	57	FAM	MN604162
JS15	F: TGACACCTCGAGATTGTGCC R: CCCGCCACCATCTTCATCTA	$(TTG)_5$	135–150	57	TAMRA	MN604161
JS16	F: CGTTAACCTGCTGCATGCTC R: CCTGAAGAAGAGTCATGTGCTG	$(CTC)_5$	193–223	56	HEX	MN604157
JS17	F: GCCACTGTCTTCACTGGGAG R: CGCAAAGGACAACAGCCAAT	$(CTT)_5$	197–221	55	HEX	MN604148
JS18	F: GGGAAGTTGTTGAAGGAGCG R: ATCACATGCGCGTTTCCTTT	$(GAT)_6$	199–211	57	HEX	MN604160
JS19	F: AGATGATTTATGGCAGCCAATGA R: TGCTGGGTAAACGCATGAGT	$(AAG)_7$	216–252	56	FAM	MN604153
JS20	F: GCAACTTGACAGTTTCTCTTCTTTG R: CTTCTCGTCATCGTCCGCAT	$(CCTCT)_5$	234–259	57	FAM	MN604167
JS21	F: ACGAACCCTGGAAGCCAAAT R: ACCACGAAGGCCTACAACTG	$(CGA)_5$	242–254	55	HEX	MN604151
JS22	F: AAAGTTGCTCCTCAGCTTGG R: TAATTAGCAATGAACAGATGGTGG	$(ATC)_7$	266–293	56	FAM	MN604147
JS23	F: TGATGGATTTCGGTTGCAACTG R: ACGCCCGAGTAGTACTCCTT	$(CTTGGT)_5$	286–304	57	HEX	MN604169
JS24	F: CGGGTCTTGCTAACTGATTTCC R: TGGGAGCTGGATTTGATCTGTC	$(TAGGAA)_5$	287–305	56	TAMRA	MN604170
JS25	F: TAGGTTTGCCGGTGTTGGAA R: CCATCTCATCTTCCAAACAACCC	$(TAT)_8$	310–334	55	HEX	MN604171
JS26	F: ACATTTGAAAGAAGACGTGTACTG R: GAGATGCAGGGATGTTTGGGA	$(AAAT)_6$	312–316	57	HEX	MN604175
JS27	F: TCTTGGCAATATGCTTCCAATCG R: TGTGTATGCTGAAGATGCTCTT	$(TTTA)_5$	312–336	55	HEX	MN604177
JS28	F: AAAGGGTGAAGGAAGAAATTAGGAT R: CCAAATTAAGCCAAACATGGTTGC	$(TCTCT)_5$	316–332	57	HEX	MN604178
JS29	F: TGCTCATTAGGAACTTACAGCT R: TGGTCTTATTCGGGTTGACACA	$(TCT)_8$	318–330	57	FAM	MN604172
JS30	F: GGACCCACGAGTGCCAAATA R: GCTTAAAGAAGTGGTAAAGTAGGTAAT	$(CCAA)_6$	319–327	55	FAM	MN604176
JS31	F: AATCACTGTTAGCCAAGCCT R: TTTGCAAATATAGAACGCCAAGA	$(TTA)_6$	321–345	57	HEX	MN604173
JS32	F: GTAGCTCTTTCAAACCGATCG R: ATCAGGAATTAGACTGAGATATTCACA	$(GAT)_6$	418–433	57	FAM	MN604174

Note: T_a = annealing temperature.

employed in previous population genetic studies (e.g., Wang et al., 2008; Gunn et al., 2010). However, our preliminary cross-species amplification results showed that most of the previously developed primers are inefficient for cultivar identification in *J. sigillata* (J. Liu et al., unpublished data). Therefore, we set out to design and screen a novel set of high-quality primers using recently published genomic data for *J. regia* (e.g., Martínez-García et al., 2016; Bai et al., 2018), with the goal of producing high-quality SSR markers capable of distinguishing taxa and cultivars within *Juglans*.

METHODS AND RESULTS

SSR detection, primer design, and validation

Genomic data of two *J. regia* individuals were downloaded from the National Center for Biotechnology Information (NCBI) database (i.e., PRJNA291087 [Martínez-García et al., 2016] and PRJNA356989 [Bai et al., 2018]). Comparing these genomes, we used QDD_v3 (Meglécz et al., 2009) to detect the SSRs with default parameters and found 279,712 loci containing microsatellites, 39,741 of which were polymorphic between the two genomes. In total, primers were designed for 14,217 of the polymorphic SSRs detected, using QDD with default parameters. After considering the PCR product size (ca. 100–400 bp), repeat type (perfect repeat), motif length (3–6 bp), and repeat frequency (>6), we selected 434 of these primers for downstream laboratory validation. We adopted a three-step pipeline to screen polymorphic primers.

Step 1: Amplification screening—Four *J. sigillata* individuals from three different populations were used to test the success of PCR amplification (Appendix 1). The PCR reaction comprised 18 μL of Golden Star T6 Super PCR Mix (TsingKe Biological Technology, Beijing, China), with 0.5 μL each of forward and reverse primers, plus 1 μL of DNA template (~50 ng DNA), in a final reaction volume of 20 μL. The PCR protocol was: 95°C for 5 min; followed by 30 cycles of 95°C for 10 s, primer-specific annealing temperatures (55–58°C, Table 1) for 3 min, 72°C for 1 min; and a final extension at 72°C for 5 min. PCRs were carried out on a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, California, USA). The obtained PCR products were detected using 6% polyacrylamide gel (PAGE), and each primer was judged to be genuine if observed

TABLE 2. Population genetic summary statistics of three *Juglans sigillata* populations.[a]

	Population												
	GD (*n* = 20)			TQT (*n* = 20)			LBT (*n* = 20)			Total (*n* = 60)	Mean		PCR amplification rate (%)
Locus	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	
JS01	2[b]	0.950	0.499	2[b]	0.950	0.499	2[b]	0.950	0.499	2	0.950	0.499	100
JS02	4	0.650	0.545	3	0.632	0.536	2	0.500	0.480	5	0.594	0.520	98
JS03	5	0.650	0.731	5	0.400	0.445	5	0.750	0.560	6	0.600	0.579	100
JS04	5	0.550	0.696	6	0.950	0.775	5[b]	0.400	0.700	6	0.633	0.724	100
JS05	3	0.650	0.565	5	0.750	0.518	3	0.550	0.526	6	0.650	0.536	100
JS06	3	0.700	0.599	3	0.600	0.595	3	0.550	0.511	3	0.617	0.568	100
JS07	2[b]	0.000	0.495	3[b]	0.000	0.395	2[b]	0.000	0.180	3	0.000	0.357	100
JS08	4	0.550	0.443	4	0.400	0.341	5	0.500	0.415	5	0.483	0.400	100
JS09	2	0.350	0.499	2	0.700	0.495	3	0.550	0.545	3	0.533	0.513	100
JS10	3	0.600	0.454	4	0.500	0.516	4	0.500	0.530	4	0.533	0.500	100
JS11	3	0.600	0.585	5	0.500	0.619	6[b]	0.500	0.686	6	0.533	0.630	100
JS12	4	0.200	0.303	4[b]	0.450	0.671	3[b]	0.400	0.559	4	0.350	0.511	100
JS13	4	0.650	0.546	4	0.800	0.636	3[b]	0.550	0.659	4	0.667	0.614	100
JS14	3[b]	0.450	0.421	3	0.700	0.579	3	0.550	0.629	3	0.567	0.543	100
JS15	2	0.100	0.095	4	0.600	0.670	5	0.600	0.574	5	0.433	0.446	100
JS16	3[b]	0.250	0.529	3[b]	0.250	0.485	3	0.550	0.551	5	0.350	0.522	100
JS17	2	0.200	0.320	2	0.350	0.469	2	0.450	0.489	2	0.333	0.426	100
JS18	4[b]	1.000	0.569	2[b]	1.000	0.500	3[b]	1.000	0.545	4	1.000	0.538	98
JS19	4[b]	0.368	0.499	6[b]	0.368	0.716	6[b]	0.400	0.713	6	0.379	0.642	97
JS20	3[b]	0.118	0.431	5[b]	0.188	0.691	4	0.588	0.678	5	0.298	0.600	83
JS21	2	0.150	0.139	3	0.250	0.301	3	0.300	0.266	3	0.233	0.235	100
JS22	6[b]	0.650	0.785	4	0.600	0.606	7	0.600	0.718	8	0.617	0.703	100
JS23	4	0.400	0.431	4	0.550	0.674	3	0.750	0.661	4	0.567	0.589	100
JS24	4	0.400	0.431	4	0.550	0.674	3	0.750	0.661	4	0.567	0.589	100
JS25	2	0.050	0.049	4	0.316	0.359	3	0.200	0.261	4	0.189	0.223	87
JS26	4	0.588	0.673	4[b]	0.474	0.669	5[b]	0.333	0.699	7	0.465	0.680	90
JS27	2	0.000	0.180	1	0.000	0.000	2[b]	0.000	0.278	2	0.000	0.153	55
JS28	2	0.444	0.346	3	0.250	0.271	4[b]	0.063	0.279	5	0.252	0.299	82
JS29	1	0.000	0.000	2[b]	0.000	0.095	2	0.050	0.139	5	0.017	0.078	100
JS30	2	0.222	0.198	2	0.313	0.264	2	0.250	0.219	2	0.262	0.227	82
JS31	4[b]	0.150	0.303	4	0.200	0.269	6[b]	0.150	0.455	2	0.167	0.342	100
JS32	1	0.000	0.000	2	0.235	0.208	2	0.100	0.095	3	0.112	0.101	85

Note: *A* = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; *n* = number of individuals.
[a]Locality and voucher information are provided in Appendix 1.
[b]Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

TABLE 3. Transferability of the 32 SSR markers developed for *Juglans sigillata* in *J. regia*, *J. cathayensis*, and *J. mandshurica*.[a]

	J. regia				*J. cathayensis*			*J. mandshurica*		
Locus	GLR (*n* = 20)	XYR (*n* = 9)	Size range (bp)	PCR amplification rate (%)	MGY (*n* = 3)	Size range (bp)	PCR amplification rate (%)	DTL (*n* = 3)	Size range (bp)	PCR amplification rate (%)
JS01	+	+	79–94	97	*	**	0	*	**	0
JS02	—	—	103	100	—	99	100	+	91–99	100
JS03	+	+	90–102	97	+	96–99	67	+	93–99	100
JS04	+	+	93–105	100	+	96–111	100	—	105	100
JS05	—	+	102–117	100	—	93	33	—	93	33
JS06	—	+	102–108	97	—	96	67	—	96	100
JS07	—	—	118	100	—	120	67	+	114–122	100
JS08	—	+	116–128	100	+	119–131	100	—	119	100
JS09	+	+	123–132	100	+	111–117	100	—	117	100
JS10	—	+	129–141	100	+	111–132	100	+	123–132	100
JS11	—	+	129–149	97	+	137–145	100	+	137–149	100
JS12	—	+	121–139	100	+	121–124	100	—	121	100
JS13	—	+	129–149	97	—	137	67	+	137–143	100
JS14	+	+	138–155	100	+	128–143	100	+	128–140	100
JS15	+	+	138–150	100	+	135–138	100	—	135	100
JS16	+	+	196–223	97	+	193–205	100	—	196	100
JS17	+	+	199–211	100	—	202	67	—	202	100
JS18	+	+	197–221	97	—	209	33	*	**	0
JS19	—	—	228–234	97	+	219–237	100	+	216–219	100
JS20	—	+	234–254	100	+	239–254	67	—	239	100
JS21	—	+	248–251	100	+	245–248	100	+	248–251	100
JS22	—	+	272–282	100	+	266–281	100	+	266–275	100
JS23	+	+	292–304	100	+	289–298	33	—	298	67
JS24	+	+	281–305	97	+	293–299	67	—	299	67
JS25	+	+	320–328	86	+	316	67	—	316	100
JS26	+	+	310–334	100	+	310–319	100	+	322–328	100
JS27	+	—	312–316	86	+	312–316	67	—	316	100
JS28	—	—	324	93	+	312–324	33	+	312–324	100
JS29	+	—	318–330	93	—	330	100	—	330	100
JS30	—	+	319–323	93	—	323	67	+	319–327	100
JS31	+	+	321–345	31	+	330–336	67	+	336–339	67
JS32	—	—	418	100	—	418	67	—	418	100

Note: — = monomorphic (only one allele was detected); + = polymorphic (two or more alleles were observed).
[a]Locality and voucher information are provided in Appendix 1.
*Unsuccessful PCR amplification.
**No result.

products were close to the expected size (ca. 20 bp fluctuation). One hundred and eleven primers successfully amplified all individuals and were further evaluated in step 2.

Step 2: Polymorphism identification—PCR amplification was performed using 20 *J. sigillata* individuals and four *J. regia* individuals from five populations (Appendix 1). Once again, the quality of the amplification products was visually evaluated using PAGE gels. Thirty-two primers showed results that were specific, reproducible, and revealed Mendelian polymorphisms and were taken forward to step 3.

Step 3: Population genetic characterization—Forward primers were 5′-end fluorescently labeled with FAM, HEX, or TAMRA (Optimus Bio, Kunming, China). Ninety-five individuals were used for the analysis, including three populations of *J. sigillata* ($n = 60$), two of *J. regia* ($n = 29$), and one each of *J. cathayensis* Dode ($n = 3$) and *J. mandschurica* Maxim. ($n = 3$) (Appendix 1). The fragment size of PCR products was determined by ABI 3730xl (Applied Biosystems). All of the 32 primer pairs selected for this stage were carried forward for data analysis.

Data analysis

Based on PCR products from step 3, genotyping was carried out using GeneMarker version 2.2.0 (SoftGenetics, State College, Pennsylvania, USA). GenAlEx version 6.5 (Peakall and Smouse, 2012) was used to calculate the number of alleles and levels of observed and expected heterozygosity. Departures from Hardy–Weinberg equilibrium were determined at locus level and population level using GENEPOP (Rousset, 2008). Finally, FSTAT version 2.9.3 (Goudet, 2001) was used to detect any linkage disequilibrium between primers. Significance levels were adjusted using a sequential Bonferroni correction for multiple comparisons.

A total of 399 primer pairs (92%) successfully amplified the target loci. The majority of loci (74.4%) were monomorphic, and 111 primer pairs (25.6%) were selected for step 2, whereas only 32 primer pairs (7.4%) were selected for population genetic screening in step 3. Of the 32 loci selected, 25 had >95% PCR amplification success rate, whereas the rate ranged from 55% to 90% for the remaining seven loci (Table 2). The number of alleles per locus ranged from two to eight, with an average of four, whereas mean levels of observed and expected heterozygosity ranged from 0.000 to 1.000 and 0.078 to 0.724, with average values of 0.436 and 0.470, respectively (Table 2). Significant deviations of Hardy–Weinberg equilibrium in terms of heterozygosity deficiency were detected in 30 of 96 locus–population pairs (Table 2). Linkage disequilibrium ($P < 0.05$) was detected between loci JS23 and JS24.

To evaluate the transferability of the newly developed markers in congeneric species, amplification was tested across 35 individuals of *J. regia*, *J. cathayensis*, and *J. mandshurica* (Appendix 1). All primer pairs successfully amplified the target loci, with the exception of locus JS01 in *J. cathayensis* and loci JS18 and JS01 in *J. mandshurica* (Table 3).

CONCLUSIONS

We developed 32 polymorphic microsatellite loci in *J. sigillata*. These primers can be used to analyze the genetic diversity and structure of *Juglans* populations, not only in *J. sigillata* but also in closely related species. Moreover, these primers can also be used to examine species boundaries and infraspecific taxonomy within the genus, thus allowing for the development of a DNA fingerprinting system. Fundamentally, these markers offer a new opportunity to solve and understand the origin and domestication history of walnut tree species.

ACKNOWLEDGMENTS

This research was funded by the National Natural Science Foundation of China (grant no. 31770367, 41571059), the Science and Technology Innovation Program of the Forestry Department of Yunnan Province ([2016]cx03), the Natural Science Foundation of Yunnan (2017FB027), and the Top-notch Young Talents Project of Yunnan Provincial "Ten Thousand Talents Program" (YNWR-QNBJ-2018-146). Molecular experiments were performed at the Laboratory of Molecular Biology at the Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

J.L., D.-Z.L., and L.-M.G. conceived and designed the experiments. J.L. collected the samples. H.-T.Q. and L.-J.Y. designed the primers. Z.-C.X. and Y.-C.J. performed the molecular laboratory work. Z.-Y.X. and Z.-C.X. analyzed the data. Z.-C.X., J.L., and R.I.M. drafted the manuscript, and all authors contributed to the manuscript revision.

DATA AVAILABILITY

Genomic data for the two *Juglans regia* specimens were downloaded from the National Center for Biotechnology Information (NCBI) database (PRJNA291087 [Martínez-García et al., 2016] and PRJNA356989 [Bai et al., 2018]). Primer sequences for the newly developed primers have been deposited to NCBI's GenBank database; accession numbers are listed in Table 1.

LITERATURE CITED

Bai, W. N., P. C. Yan, B. W. Zhang, K. E. Woeste, K. Lin, and D. Y. Zhang. 2018. Demographically idiosyncratic responses to climate change and rapid Pleistocene diversification of the walnut genus *Juglans* (Juglandaceae) revealed by whole-genome sequences. *New Phytologist* 217: 1726–1736.

Cooke, R. J. 1995. Introduction: The reasons for variety identification. *In* C. W. Wrigley [ed.], Identification of food grain varieties, 1–18. American Association of Cereal Chemists, Eagan, Minnesota, USA.

Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Institute of Ecology, Lausanne, Switzerland. Website http://www2.unil.ch/popgen/softwares/fstat.htm [accessed 2 February 2020].

Gunn, B. F., M. Aradhya, J. M. Salick, A. J. Miller, Y. P. Yang, L. Liu, and X. Hai. 2010. Genetic variation in walnuts (*Juglans regia* and *J. sigillata*; Juglandaceae): Species distinctions, human impacts, and the conservation of agrobiodiversity in Yunnan, China. *American Journal of Botany* 97: 660–671.

Hoban, S., R. Anderson, T. I. M. McCleary, S. Schlarbaum, and J. Romero-Severson. 2008. Thirteen nuclear microsatellite loci for butternut (*Juglans cinerea* L.). *Molecular Ecology Resources* 8: 643–646.

Hodel, R. G., M. C. Segovia-Salcedo, J. B. Landis, A. A. Crowl, M. Sun, X. Liu, M. A. Gitzendanner, et al. 2016. The report of my death was an exaggeration: A

review for researchers using microsatellites in the 21st century. *Applications in Plant Sciences* 4: 1600025.

Ikhsan, A. S., H. Topçu, M. Sütyemez, and S. Kafkas. 2016. Novel 307 polymorphic SSR markers from BAC-end sequences in walnut (*Juglans regia* L.): Effects of motif types and repeat lengths on polymorphism and genetic diversity. *Scientia Horticulturae* 213: 1–4.

Lu, A., D. Stone, and L. Grauke. 1999. Juglandaceae. *In* Z. Y. Wu and P. H. Raven [eds.], Flora of China, Vol. 4, 277–285. Science Press, Beijing, China, and Missouri Botanical Garden Press, St. Louis, Missouri, USA.

Martínez-García, P. J., M. W. Crepeau, D. Puiu, D. Gonzalez-Ibeas, J. Whalen, K. A. Stevens, R. Paul, et al. 2016. The walnut (*Juglans regia*) genome sequence reveals diversity in genes coding for the biosynthesis of non-structural polyphenols. *Plant Journal* 87: 507–532.

Meglécz, E., C. Costedoat, V. Dubut, A. Gilles, T. Malausa, N. Pech, and J.-F. Martin. 2009. QDD: A user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* 26: 403–404.

Peakall, R., and P. E. Smouse. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research–an update. *Bioinformatics* 28: 2537–2539.

Powell, W., G. C. Machray, and J. Provan. 1996. Polymorphism revealed by simple sequence repeats. *Trends in Plant Science* 1: 215–222.

Rousset, F. 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

Wang, H., D. Pei, R. S. Gu, and B. Q. Wang. 2008. Genetic diversity and structure of walnut populations in central and southwestern China revealed by microsatellite markers. *Journal of the American Society for Horticultural Science* 133: 197–203.

Woeste, K., R. Burns, O. Rhodes, and C. Michler. 2002. Thirty polymorphic nuclear microsatellite loci from black walnut. *Journal of Heredity* 93: 58–60.

Zane, L., L. Bargelloni, and T. Patarnello. 2002. Strategies for microsatellite isolation: A review. *Molecular Ecology* 11: 1–16.

APPENDIX 1. Locality and voucher information for populations of *Juglans sigillata*, *J. regia*, *J. cathayensis*, and *J. mandshurica* used in the present study.

Species	Population code	Location (County, province, country)	Latitude (°N)	Longitude (°E)	Elevation (m)	*N*	Voucher specimen[a]
J. sigillata Dode	GD	Gongshan, Yunnan, China	27.916608	98.339602	2100	20	LiuJ9781–LiuJ9800
J. sigillata	TQT	Tengchong, Yunnan, China	25.255069	98.700549	2200	20	LiuJ9761–LiuJ9780
J. sigillata	LBT	Longyangqu, Yunnan, China	25.302984	98.791248	1950	20	LiuJ9809–LiuJ9828
J. regia L.	GLR	Gongliu, Xinjiang, China	43.353333	82.835417	1322	20	16CS12110
J. regia	XYR	Xinyuan, Xinjiang, China	43.521333	83.939666	1250	9	LiuJ167917–LiuJ167925
J. cathayensis Dode	MGY	Meigu, Sichuan, China	29.352999	103.53233	2145	3	LiuJ9844–LiuJ9846
J. mandshurica Maxim.	DTL	Tonghua, Jilin, China	42.511167	125.91467	513	3	LiuJ167742–LiuJ167744

[a]Voucher specimens have been deposited at the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN).